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Brief report
A simplified method of determining synovial fluid chondroitin sulfate chain length

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Summary

Objective: To determine whether dimethylmethylene blue (DMMB) analysis, when combined with agarose gel filtration chromatography (Superose 6), can be performed instead of fluorophore-assisted carbohydrate electrophoresis (FACE) to determine chondroitin sulfate (CS) chain length in synovial fluid (SF).

Methods: SF was obtained from (1) normal horses after 8 weeks of rest, (2) the same horses after 9 months of treadmill training, and (3) horses with osteochondral (OC) injury from racing. SF CS concentrations and chain lengths were determined by gel chromatography and DMMB analysis and compared with previous results determined by FACE analysis on the same samples.

Results: DMMB analysis showed that SF CS peak chain length in the OC injury group increased significantly (18.7 kDa) when compared to rested and exercised normal horses (15.6 kDa). The assay had a positive predictive value of 71% and a negative predictive value of 75% for discriminating between normal and injured joints.

Conclusions: We report a simple and inexpensive DMMB analysis of SF CS chain length, which, when coupled with Superose 6 chromatography, discriminates between normal and post-injury joints. Similar to our previous FACE analysis results [Brown MP, Trumble TN, Plaas AHK, Sandy JD, Romano M, Hernandez J, *et-al.* Exercise and injury increase chondroitin sulfate chain length and decrease hyaluronan chain length in synovial fluid. Osteoarthritis Cartilage 2007;15], our DMMB results show an increase in the chain length of the CS in the SF of injured joints.

Key words: Chondroitin sulfate chain length, Dimethylmethylene blue, Fluorophore-assisted carbohydrate electrophoresis, Synovial fluid, Biomarkers, Joint injury, Horse.

Introduction

Chondroitin sulfate (CS) has been widely studied as a biomarker to monitor changes in the metabolism of joints[2–7]. We have shown previously, by sequential use of agarose gel filtration chromatography (Superose 6) and fluorophore-assisted carbohydrate electrophoresis (FACE), that CS chains in synovial fluid (SF) from injured joints are significantly longer than those in normal joints[1]. Thus, determination of SF CS chain length has potential application as a biomarker of joint injury. Unfortunately, FACE is a technique that requires specialized laboratory equipment and a high level of technical expertise. Additionally, FACE technique is expensive in terms of materials and time per sample. The aim of the present study was to determine whether dimethylmethylene blue (DMMB) analysis could be used instead of FACE to determine CS chain length in SF. If so, the more practical DMMB would make determination of SF CS chain length much more feasible as a research or clinical screening tool.

Methods, results and discussion

SF was obtained from carpal joints of (1) eight normal Thoroughbred horses (3–6 years of age) after 8 weeks of rest, (2) the same horses after 9 months of treadmill training, and (3) seven Thoroughbred horses (3–6 years of age) with osteochondral (OC) injury from racing. Specifically, OC injuries are small OC fracture fragments from the dorsal articular margins of the carpal bones and are traumatic in origin. The protocol was approved by the Institutional Animal Care and Use Committee. Superose 6 chromatography and FACE analysis had previously been performed on these samples[1].

Aliquots (250 μl) of SFs were digested with proteinase K (1 mg proteinase K/sample; GIBCO, Carlsbad, CA), followed by enzyme inactivation at 100°C for 10 min and filtration through a 0.45 μm nylon membrane. Samples were then fractionated on gel filtration columns (HR 10/30; GE Healthcare, Pittsburgh, PA) packed with agarose (Superose 6; GE Healthcare) as previously described[1]. Column

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effluent was collected in 1 ml fractions by using a fraction collector (GE Healthcare). Fractions 1 through 13 were discarded and fractions 14 through 23 were collected for DMMB analysis (10 fractions per joint). The Superose 6 column was previously calibrated for CS molecular weight[8]. Our previous work has shown that the sulfated glycosaminoglycans (GAGs) contained in these fractions are CS[1]. Fractions (1 ml) were vacuum dried and reconstituted in 100 μl of water. DMMB was performed by modification of the Blyscan® method (Biocolor, Accurate Chemical Supplies, Westbury, NY), adapted to a 384-well microplate technique. Blyscan® dye reagent (1 ml) was added to each sample, shaken for 30 min, then centrifuged (13,000 *g*) for 20 min. Supernatant was decanted with care not to disturb the precipitate. Tubes were inverted on paper towel and allowed to drain. Blyscan® dissociation reagent was added based on the anticipated amount of GAGs in the sample (300 μl for fractions 14 through 16 and 21 through 23; 600 μl for fractions 17 through 20), and the sample vortexed briefly. Chondroitin-4-sulfate standards (Biocolor) and samples (60 μl aliquots) were pipetted into a 384-well microplate (Fisher, Pittsburgh, PA) and absorbance was read at 650 nm, using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Inc., Durham, NC).

Average molecular weights (kDa) of CS chains in individual fractions from the Superose 6 column were estimated by the relationship between the experimentally determined number of repeating disaccharides and the partition coefficient (K_D) for the column used, as previously described[8]. Partition coefficient (K_D) was estimated from

$$K_D = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the fraction, V_0 is the void volume of the column and V_t is the total volume of the column[9]. The molecular mass of the typical CS disaccharide unit was assumed to be 446 Da[8].

The DMMB assay measures total sulfated GAGs[10]. Therefore, in order to compare DMMB results directly with FACE results, we used total sulfated chondroitin disaccharide (Δdi6S and Δdi4S) determined by FACE for the same samples[1]. Values for non-sulfated chondroitin disaccharide (Δdi0S) from the FACE analysis were excluded from the comparison because DMMB does not detect non-sulfated GAGs[10]. Area under the curve (AUC) for total GAG concentration was compared between the two assay methods by the Wilcoxon matched-pairs signed-ranks test, with $P < 0.05$ considered significant. Fisher's exact test was used to determine the sensitivity, specificity, positive predictive value, and negative predictive value of using the SF CS chain length to discriminate between OC injured and normal (rested and exercised values combined) joints.

Sulfated GAG from SF was quantitated in μg/ml of fluid by DMMB, according to fragment size (Superose 6 fraction number). Figure 1(A) shows mean (+SD) SF CS content of the three groups as determined by DMMB. For comparison, Fig. 1(B) shows mean (+SD) total sulfated chondroitin disaccharide (Δdi6S and Δdi4S) for the same samples determined by FACE. For both methods, the CS peak occurred at fraction #18 (18.7 kDa) in SF from joints with OC injury. Similarly, CS in SF from exercised joints peaked in fraction #19 (15.6 kDa) with both assays. CS in SF from rested joints peaked in fraction #19 (15.6 kDa) when determined by DMMB, but peaked in fraction #20 (11.6 kDa) when measured by FACE [Fig. 1(A) and (B)]. When AUC values were compared between the two assay methods

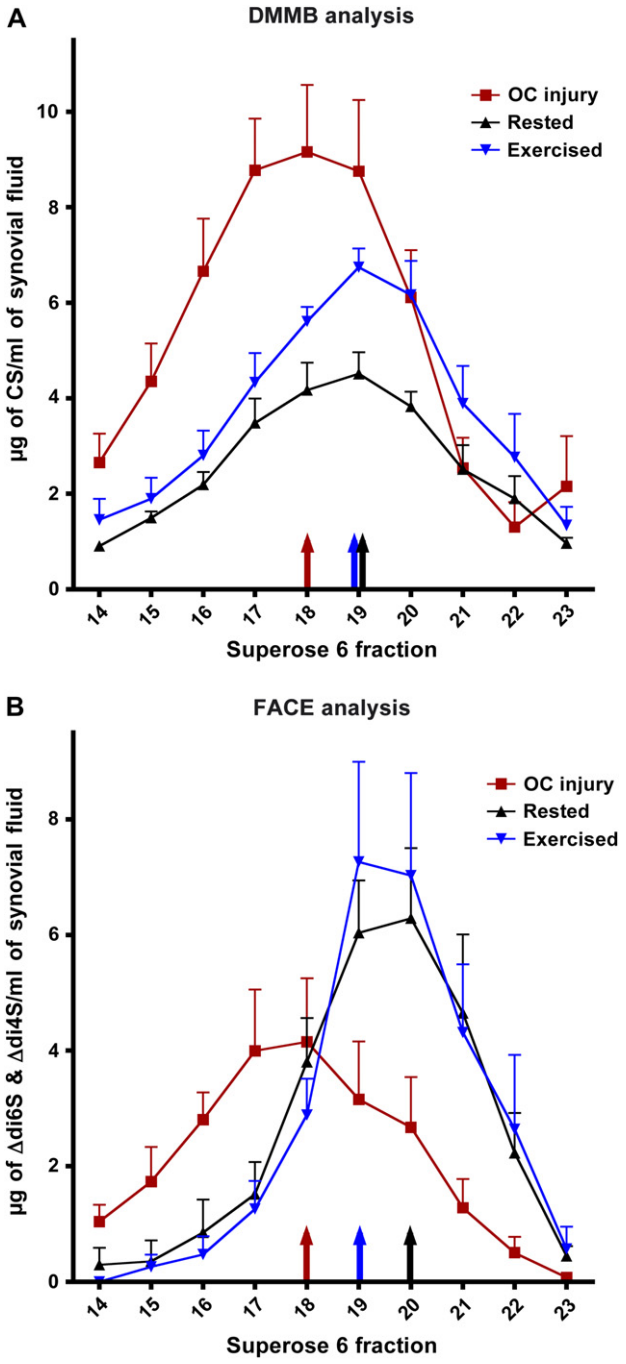

Fig. 1. (A) Mean (+SD) CS concentrations in fractions of SF from rested and exercised horses with normal joints and from horses with OC injury. (B) Mean (+SD) total sulfated chondroitin disaccharide (Δdi6S and Δdi4S) for the same samples determined by FACE. Superose 6 fraction at which peak occurs for OC injury (red arrows), rested (black arrows), and exercised (blue arrows).

for the OC injury and exercised groups, the DMMB technique yielded significantly higher AUC values than FACE. However, the two assays yielded similar AUC values for the rested group (Table I).

Low volumes of DMMB reagents could be used with a 384-well microplate, facilitating quantitation of the small amounts of CS in fractions collected from Superose 6 chromatography. When used for quantitation of sulfated CS in

Table I
AUC (±SD) for each group and assay (μg of CS/ml of SF)

Assay	OC injury*	Rested	Exercised*
DMMB	52.5 ± 3.0	24.3 ± 1.3	43.3 ± 2.4
FACE	21.3 ± 1.4	26.5 ± 2.4	28.6 ± 2.9

*AUC values for OC injury and exercised joints were significantly different between assays ($P < 0.0001$), but values for rested joints were not ($P = 0.92$).

SF (μg/ml), DMMB results were significantly higher than those from FACE analysis. Although it is possible that DMMB may be measuring other sulfated GAGs in addition to CS, this is unlikely, because our previous FACE analysis results have shown that CS and hyaluronan are the only GAGs in these Superose 6 fractions[1]. Keratan sulfate is eluted from the column in later fractions (data not shown) and therefore should not be contributing to sulfated GAGs measured by the DMMB assay. Therefore, we expect that CS is the only sulfated GAG being measured by DMMB in these fractions. Similar to our previous FACE analysis results[1], our DMMB results show an increase in the chain length of the CS in the SF of OC injured joints when compared to rested and exercised horses.

Although the Superose 6/DMMB assay has low sensitivity, its specificity is higher and more comparable to the FACE assay in which Δdi6S and Δdi4S values were combined (Table II). The usefulness of an assay is often defined by its positive and negative predictive values. Positive predictive value gives the probability that a joint classified as positive by the assay is indeed injured. Conversely, negative predictive value gives the probability that a joint that is classified as negative by the assay is really normal. These values were calculated by using Superose 6 fraction ≤ 18 as a breakpoint for OC injured joints and Superose 6 fraction ≥ 19 as a breakpoint for normal joints (rested and exercised values combined). This yielded a positive predictive value of 71% and a negative predictive value of 75% for the DMMB assay, compared with a positive predictive value of 86% and a negative predictive value of 94% for the FACE assay when values for Δdi6S and Δdi4S were combined to allow a direct comparison between the assays. Improved predictive values were seen for FACE when each disaccharide was considered separately in a previous study[1].

In summary, when combined with Superose 6 chromatography, DMMB assay was less accurate than FACE in discriminating between SF from OC injured and normal joints. The lower accuracy of DMMB is probably related to the fact that the measured sulfated GAG (presumed to be CS) is less well defined than the CS disaccharides measured by FACE. However, the DMMB assay is much more practical than FACE because it requires less time, expense, and technical expertise. Thus, it would be more readily adapted to routine use and may be useful as a screening tool for both research and clinical applications. Therefore, our described DMMB microplate assay, when coupled with Superose 6 chromatography, may provide a simple and inexpensive tool for determining CS chain length in evaluation of joint health.

Table II
Sensitivity, specificity, positive predictive value, and negative predictive value of DMMB and FACE assays of SF CS chain length to discriminate between OC injured (≤Superose fraction B18) and normal (≥Superose fraction B19; rested and exercised values combined) joints

Assay	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
DMMB	56	86	71	75
FACE	86	94	86	94

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